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Immunogenicities of intravenous and intramuscular administrations of modified vaccinia virus Ankara-based multi-CTL epitope vaccine for human immunodeficiency virus type 1 in mice

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A vaccine against human immunodeficiency virus (HIV) is still awaited. Although the correlates of protection remain elusive, it is likely that CD8 T cells play an important role in the control of this infection. To firmly establish the importance of these cells in protective immunity, a means of efficient elicitation of CD8 T cell responses in the absence of antibody is needed and, when available, might represent a crucial step towards a protective vaccine. Here, a novel vaccine candidate was constructed as a multi-cytotoxic T lymphocyte (CTL) epitope gene delivered and expressed using modified vaccinia virus Ankara (MVA). The immunogen consists of 20 human, one murine and three rhesus macaque epitopes. The non-human epitopes were included so that the vaccine can be tested for immunogenicity and optimal vaccination doses, routes and regimes in experimental animals. Mice were immunized intravenously (i.v.) or intramuscularly (i.m.) using a single dose of 10^6 p.f.u. of the recombinant MVA and the induction of CTL was assessed. It was demonstrated that both administration routes induced specific CTL responses and that the i.v. route was moderately more immunogenic than the i.m. route. The frequencies of *ex vivo* splenocytes producing interferon-γ upon MHC class I-restricted peptide stimulation were determined using an ELISPOT assay. Also, the correct processing and presentation of some HLA-restricted epitopes in human cells was confirmed.

Introduction

$CD8^+$ cytotoxic T lymphocyte (CTL) activity is an important component of the organism's defence against many virus diseases (McMichael, 1992). To induce and be recognized by CTL, antigen-presenting cells degrade virus-derived antigens into peptide epitopes eight to ten amino acid residues in length which are displayed on the cell surface in association with major histocompatibility complex (MHC) class I molecules (Zinkernagel & Doherty, 1975). The fact that only small parts of antigens are sufficient for induction of CTL has been exploited in the construction of polyepitope-based vaccines (Hanke *et al.*, 1997*a*, *b*; Thomson *et al.*, 1995, 1996; Yasutomi *et al.*, 1995). Such an approach minimizes the amount of administered protein and enables the immune response to be focused on important or highly conserved epitopes. The variability in the sets of peptides against which individuals can produce a CTL response can be accommodated by incorporating epitopes restricted by many different human leukocyte antigen (HLA) alleles. It has been estimated that five optimally selected MHC class I-restricted epitopes would enable 80–90% of the Caucasian and Oriental populations to mount a CTL response, and eight to nine peptides would be required for a coverage of the general population (Lalvani *et al.*, 1994; Sidney *et al.*, 1996).

Antigens enter the MHC class I-processing pathway most efficiently during their intracellular synthesis. Therefore im-

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munization vectors that deliver virus genes into the host cells are the most promising vaccine vehicles for effective induction of CTL. One such vector is modified vaccinia virus Ankara (MVA) (Mayr *et al.*, 1975). This is a highly attenuated strain of vaccinia virus which grows to high titres in chicken embryo fibroblasts (CEF), but has virtually lost its ability to multiply in mammalian cells. During the life-cycle in human cells, MVA growth is restricted at the stage of virion formation, while DNA replication and both early and late protein synthesis appear to be normal (Sutter & Moss, 1992). The attenuation may be partly attributable to six major genome deletions totalling approximately 31000 bp (Altenburger *et al.*, 1989; Meyer *et al.*, 1991) and the appropriate profile of virus-encoded cytokine receptors: MVA expresses an interleukin-1β receptor, but lacks functional receptors for interferon (IFN)-α/β, IFN-γ and tumour necrosis factor (T. J. Blanchard and others, unpublished). MVA was shown to be avirulent in immunosuppressed animals and, most importantly, to be safe with no adverse side-effects when used in over 120000 humans at the end of the smallpox eradication campaign (Mayr *et al.*, 1975).

Development of an anti-human immunodeficiency virus (HIV) vaccine is one of the major goals of research with AIDS. Although no uniform guiding principles for the requirements for protection are available, there is ample rationale for the importance of cellular immunity, including CTL, in the control of HIV infection (Carmichael *et al.*, 1993; Kalams *et al.*, 1994; Plata *et al.*, 1987; Rowland-Jones *et al.*, 1993, 1995; Walker *et al.*, 1987). In order to test the role of CTL more rigorously, a means of reliable CTL induction in the absence of antibody is needed. Successful development of such a procedure might at the same time represent a significant step towards a vaccine either preventing HIV infection or attenuating it to prevent disease.

As a part of the development of an anti-HIV vaccine, a multi-CTL epitope gene termed H was constructed (Hanke *et al.*, 1997*a*). It was derived from human and simian immunodeficiency virus sequences and contains 20 human, one murine and three macaque epitopes. The non-human epitopes were included so that the vaccine can be tested for optimal vaccination doses, routes and regimes in experimental animals, which will allow effective planning of trials in humans. It was shown that immunization of mice using the H polyepitope expressed from a DNA vector alone or coupled to *Plasmodium*-derived epitopes (also containing one murine epitope), designated HM, efficiently induced epitope-specific CTL responses (Hanke *et al.*, 1997*a*). Here, MVA is explored as an alternative vehicle for a multi-CTL epitope AIDS vaccine candidate. Prior to immunization studies in primates, mice were inoculated using intramuscular (i.m.) and intravenous (i.v.) routes, and the induction of CTL responses directed against both the HIV and *Plasmodium* murine epitopes was determined. The MVA.H and MVA.HM vectors also permitted confirmation of the correct processing of several HLA-restricted epitopes from the multi-epitope proteins in human cells.

Methods

■ **Cells.** COS-1 and CEF cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% foetal bovine serum (FBS; Gibco) and penicillin/streptomycin (P/S). The P815 mastocytoma cell line was grown in RPMI 1640 (Gibco) supplemented with 10% FBS and P/S. All cells were cultured in humidified incubators in 5% CO_2 at 37 °C.

■ **Antibodies.** MAb SV5-P-k specific for the Pk epitope (Serotech) has been described previously (Hanke *et al.*, 1992). R4 (ATCC) and biotin-conjugated XMG1.2 (Pharmigen) MAbs were used in the enzyme-linked immunospot (ELISPOT) assays for detection of murine IFN-γ. For lymphocyte panning, rat anti-mouse CD4 KT174 and rat anti-mouse CD8α KT15 MAbs (Serotech) were employed.

■ **Preparation of recombinant MVA.** The parental MVA was a gift from A. Mayr, University of Munich (Mayr *et al.*, 1978). To generate recombinant MVA, the H and HM polyepitope genes were excised from plasmids pTH.H and pTH.HM (Hanke *et al.*, 1997*a*) using *Hin*dIII and *Pst*I restriction endonucleases, the staggered ends filled in with the Klenow fragment of DNA polymerase and the blunt-ended fragments inserted into the *Sma*I site of pSC11 (Chakrabarti *et al.*, 1985). Plasmids carrying the heterologous genes were transfected into CEF cells which had been infected 1 h earlier with MVA at 0·05 p.f.u. per cell, and *in vivo* homologous recombination directed the H and HM genes into the thymidine kinase locus of MVA. The total virus from the cells and supernatant was harvested 3 days later and used for re-infection of CEF monolayers. The plaques of recombinant viruses were identified using an X-Gal colour selection (Carroll & Moss, 1995; Chakrabarti *et al.*, 1985) and purified by five rounds of plaque purification. Bulk stocks of the recombinant viruses were grown and purified by centrifugation of cytoplasmic extracts through a 36% (w/v) sucrose cushion in a Beckman SW28 rotor at 13500 r.p.m. for 80 min. The purity of the recombinant viruses was verified by PCR analysis across the thymidine kinase gene locus and by the fact that all plaques produced blue colour upon incubation with X-Gal.

■ **MVA infection of CEF, COS-1 and 293T cells.** Cell monolayers were grown to approximately 70% confluency and infected with recombinant MVA at various m.o.i. values. Cells were harvested 16 h later, washed with PBS, resuspended in a disruption buffer (5% glycerol, 50 mM Tris–HCl pH 7, 2% SDS, 5% 2-mercaptoethanol, 0·25% bromophenol blue), boiled and stored at −20 °C.

■ **SDS–PAGE and Western blot analysis.** Individual polypeptides in infected cell lysates were separated on an SDS–polyacrylamide gel cross-linked with 15% *N,N*-diallyltartardiamide using the Hoefer Scientific System electrophoresis set. Separated polypeptides were transferred onto a nylon filter (Amersham) using a semi-dry gel electroblotter (LKB). The filters were blocked with PBS + 20% (w/v) skimmed milk and incubated with SV5-P-k MAb in PBS + 5% (w/v) skimmed milk. Bound antibodies were detected using horseradish peroxidase (HRP)-conjugated protein A (Amersham) in PBS + 5% (w/v) skimmed milk followed by enhanced chemiluminescence (Amersham).

■ **Mouse immunization.** BALB/c mice were immunized either i.m. or i.v. with 10^6 p.f.u. of MVA. For the i.m. route, mice were anaesthetized and the calf muscles of each lower hind leg were injected with MVA in 50 µl sterile PBS. For the i.v. route, mice were heated in a stream of 37 °C warm air, restrained and injected with MVA in 200 µl sterile PBS into the lateral tail vein.

■ **CTL cultures.** At two time-points after immunization, spleens were removed and pressed individually through a cell strainer (Falcon) using a 2 ml syringe rubber plunger. The splenocytes were washed twice,

suspended in 10 ml lymphocyte medium (RPMI 1640 supplemented with 10% FBS, P/S, 20 mM HEPES and 15 mM 2-mercaptoethanol) and incubated with 2 μg/ml peptide RGPGRAFVTI (Takahashi *et al.*, 1993) or together with SYIPSAEKI (Romero *et al.*, 1989) in a humidified incubator in 5% CO_2 at 37 °C for 5 days.

■ **Anti-CD4 and anti-CD8 lymphocyte panning.** Petri dishes (15 mm) were coated with either anti-mouse CD4 (Serotech) or anti-mouse CD8α (Serotech) MAb at 10 μg/ml in PBS at 4 °C overnight. Next day, wells were washed three times with PBS and approximately 30×10^6 freshly isolated splenocytes were added and incubated in 5% CO_2 at 37 °C for 1 h. The unattached cells were then gently removed and used in the ^{51}Cr-release assay.

■ **Target cells and standard ^{51}Cr-release assay.** The effector cells were double-diluted in U-bottom wells (96-well plate; Costar) to yield effector-to-target ratios from 100:1 to 12:1. Five thousand ^{51}Cr-labelled P815 cells in a medium containing 10^{-7} M peptide was then added to the effectors and incubated at 37 °C for 4 h. Spontaneous and total chromium releases were estimated from wells in which the target cells were kept in a medium alone or with 5% Triton X-100, respectively. Percentage specific lysis was calculated as [(sample release − spontaneous release)/(total release − spontaneous release)]x100. The spontaneous release was lower than 20%. For the net percentage release, the no-peptide background counts were subtracted from the assay well counts.

■ **ELISPOT assay.** The ELISPOT assay for detection of IFN-γ-releasing cells upon specific peptide stimulation has been described previously (Miyahira *et al.*, 1995). Nitrocellulose-backed plates (96-well, MAHA S45; Millipore) were coated with 50 μl of 15 μg/ml murine IFN-γ-specific MAb R4 (ATCC) overnight at 4 °C, washed six times with PBS and blocked using a medium supplemented with 10% FBS at room temperature for 1 h. Three dilutions ($3x10^5$, $1x10^5$ and $0{\cdot}3x10^5$) of freshly isolated splenocytes and 2 μg/ml of a specific peptide were then added into the wells and incubated overnight at 37 °C in 5% CO_2. $3x10^5$ cells without peptide were incubated as a negative control. The cells were washed three times with PBS, then 1 μg/ml of secondary biotin-conjugated antibody XMG1.2 (Pharmigen) was added and reacted at room temperature for 3 h. The wells were washed six times with PBS, and alkaline phosphatase (AP)-labelled streptavidin (Sigma) was added at 1:1000 dilution for 1 h. The wells were washed again six times with PBS and the spots were developed by adding the AP substrate 3,3′-diaminobenzidine-tetrahydrochloride dihydrate (Sigma). After 15 min the wells were washed with tap water, dried, and the spots counted under a dissection microscope.

■ **Human CTL assays.** B lymphoblastoid cell lines expressing desired HLA molecules were infected overnight with either MVA.H or MVA.HM at an m.o.i. of 25, ^{51}Cr-labelled, and tested for sensitivity to a specific lysis by established CTL clones or lines in a CTL assay. MVA.NP, which expresses the influenza virus nucleoprotein, was used a negative control. The percentage specific lysis was calculated as described above for the murine assays.

Results

MVA.H and MVA.HM isolations and preparation of virus stocks

The assembly of multi-CTL epitope genes H and HM has been described previously (Hanke *et al.*, 1997*a*). Briefly, the H protein contains 20 human (restricted by 12 different HLA molecules), one murine and three rhesus macaque CTL epitopes. The human epitopes were derived from HIV-1 clade B, which is the most common European/North American clade, and the CTL responses specific for these epitopes were detected in HIV-infected individuals. The design of the H protein included several partially overlapping epitopes to minimize the immunogen size. In polypeptide HM, the H epitopes were coupled at their C terminus to 'malaria' epitopes that included one murine epitope of *Plasmodium berghei* and eight human epitopes of *P. falciparum* (Gilbert *et al.*, 1997). The HM construct was used in this study to demonstrate the ease of building up multi-epitope vaccines and also because the immunogenicities of two murine epitopes in parallel could be assessed.

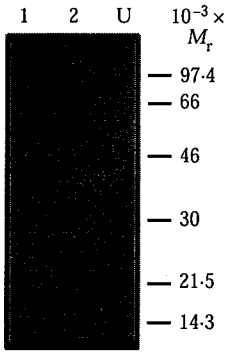

Fig. 1. Detection of the H and HM polypeptides in MVA.H- and MVA.HM-infected CEF cells. Monolayers of CEF cells were infected at an m.o.i. of 10 with MVA.H (lane 1) or MVA.HM (lane 2), or left uninfected (lane U). Cells were harvested 16 h later, lysed, the polypeptides separated by SDS–PAGE (15% gel) and Western blotted, and the recombinant proteins detected using a MAb specific for the C-terminal Pk tag followed by HRP-conjugated protein A and enhanced chemiluminescence. The positions of relative molecular mass markers are indicated on the side.

The parental virus used for construction of the recombinant MVA.H and MVA.HM viruses was MVA batch II/85 (Mayr *et al.*, 1978). Using *in vivo* homologous recombination, the H and HM genes together with the early/late P7.5 promoter (Mackett *et al.*, 1984) controlling their transcription were inserted into the thymidine kinase locus of MVA. After five rounds of plaque purification, bulk stocks of MVA.H and MVA.HM viruses were grown (no more than four passages) and partially purified as described under Methods. Both parental and recombinant MVAs were propagated on primary CEF cells obtained from the eggs of a specific pathogen-free flock.

Expression of H and HM polypeptides in MVA-infected cells

Prior to immunization of experimental animals, the expression of recombinant polyepitopes from MVA.H and MVA.HM was assessed *in vitro*. Both the H and HM polypeptides have a tag Pk of nine amino acid residues (Hanke *et al.*, 1992) attached to their C termini, which is recognized by a MAb (Hanke *et al.*, 1997*a*). Following infection of CEF cells, HM but not H could be detected by Western blot analysis using the Pk-specific MAb (Fig. 1). The HM protein migrated

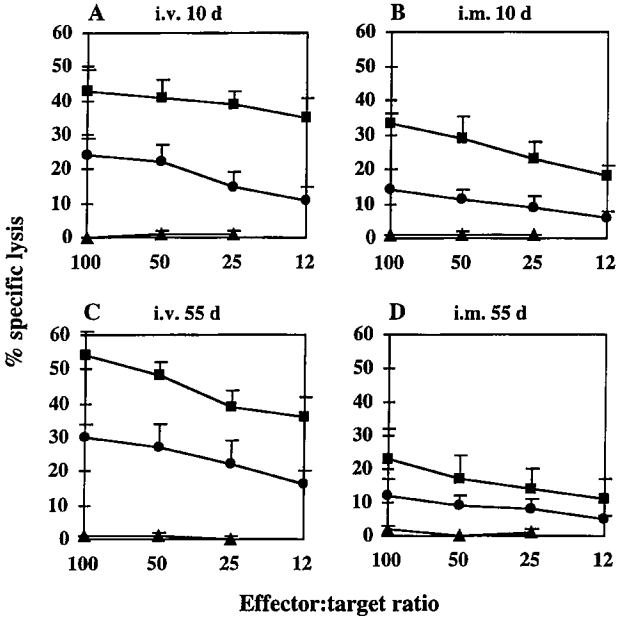

Fig. 2. Induction of CTL by MVA.HM vaccination. Groups of five female BALB/c mice were immunized i.v. (A, C) or i.m. (B, D) with 10^6 p.f.u. MVA.HM, sacrificed 10 (A, B) or 55 (C, D) days later, and the CTL responses against both the HIV (■) and *Plasmodium* (●) epitopes were determined separately for each animal. Lysis of peptide unpulsed P815 cells is also shown (▲). Each point represents the average lysis ± SD.

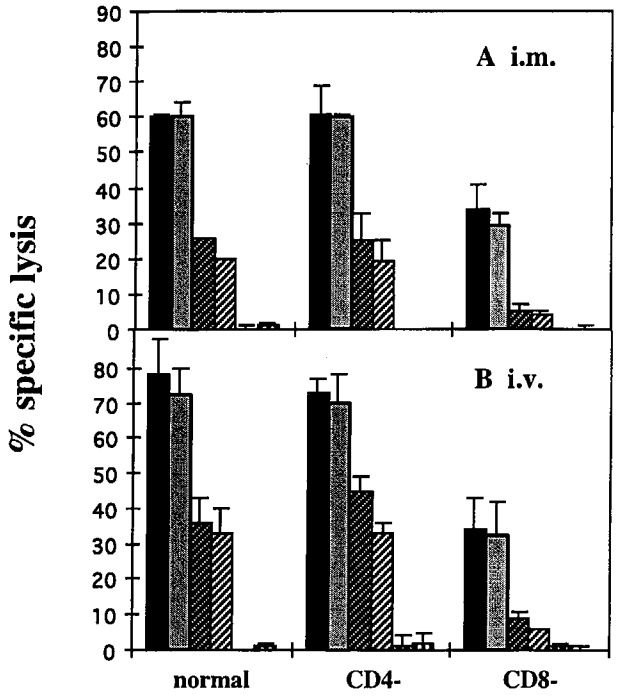

Fig. 3. Cytotoxic T cells are predominantly $CD8^+$. Groups of mice were vaccinated i.m. (A) or i.v. (B) with 10^7 p.f.u. MVA.HM and sacrificed 10 days later. Their splenocytes were isolated and restimulated *in vitro* with a mixture of the HIV and *Plasmodium* peptides for 5 days. The splenocytes were then incubated on antibody-coated plates to remove either $CD4^+$ or $CD8^+$ lymphocytes and the unattached cells were used in a ^{51}Cr-release assay against HIV (first and second bars in each group), *Plasmodium* (third and fourth bars in each group) or no (fifth and sixth bars in each group) peptide-pulsed P815 cells. The effector to target ratios were 100:1 (odd bars) and 50:1 (even bars) and the bars represent the average lysis ± SD of duplicate wells of two mice.

at approximately 25 kDa corresponding to the protein's predicted molecular mass. No expression of either H or HM polypeptides was detected in COS-1 and 293T cell lines, which are non-permissive for MVA replication, even at an m.o.i. as high as 100. In contrast, the same proteins were readily detected following transient transfection of these cells with plasmid DNA (Hanke *et al.*, 1997*a*). With both the DNA and MVA, the HM was more abundant than the H polypeptide.

Induction of CD8 T cells by i.m. and i.v. infection

It is important for newly developed vaccines to be tested for safety, immunogenicity and optimal vaccination schedule in experimental animals. For this reason, one murine and three rhesus macaque epitopes were included in the H polypeptide. The murine epitope RGPGRAFVTI presented by the H-$2D^d$ molecule originates in the V3 region of the HIV-1 envelope (Takahashi *et al.*, 1993). Epitope SYIPSAEKI (previously designated pb9) in the M part of HM is derived from the circumsporozoite protein of *P. berghei* and restricted by the H-$2K^d$ molecule (Romero *et al.*, 1989).

There are several routes that can be used for vaccination with live attenuated viruses. In this work, the i.v. and i.m. routes were evaluated: the i.v. route may induce immune responses in animals most efficiently, and the i.m. route is a suitable route for humans. The induction of CTL responses was assessed after a single vaccination dose of 10^6 p.f.u. of recombinant MVA. Firstly, CTL elicited using MVA.H expressing the HIV-derived polyepitope alone were analysed in groups of three mice. The splenocytes were isolated 10 days post-vaccination, restimulated *in vitro* with the HIV peptide for 5 days and tested for lysis of P815 cells pulsed with the same peptide in a standard ^{51}Cr-release assay. Following i.v. administration, the specific ^{51}Cr release at an effector-to-target ratio of 100:1 (E:T 100) was 50, 50 and 43% for the three animals. The i.m. route appeared to be less efficient for induction of CTL and resulted in specific ^{51}Cr release of 31, 19 and 12% at E:T 100 (data not shown). The killing of target cells that had not been pulsed with peptide was 0–1%. A 5 day peptide restimulation *in vitro* of unprimed splenocytes failed to induce specific CTL activity (data not shown).

In the next experiment, groups of five mice were immunized with MVA.HM expressing the combined HIV and *Plasmodium* polyepitope, again using both i.v. and i.m. routes, and the CTL responses induced by the two murine epitopes were determined at two different times post-infection. Following i.v. infection, the *in vitro* lytic activity specific for the HIV and *Plasmodium* peptides averaged 43 and 24% specific ^{51}Cr release, respectively, at 10 days post-infection (Fig. 2A) and

Table 1. ELISPOT assay-determined frequencies of peptide-stimulated IFN-γ-producing cells generated by MVA.HM vaccination

Mice were vaccinated with 10^6 infectious units of MVA.HM.

Route of vaccination	Time (days post-immunization)	Frequencies* HIV peptide	Frequencies* Plasmodium peptide
i.v.	10	283±27	130±53
i.v.	55	133±83	90±37
i.m.	10	227±89	148±49
i.m.	55	60±13	40±17

* Frequencies are shown as the mean number ±SD of responding cells per 10^6 splenocytes.

increased to 54 and 30%, respectively, by day 55 (Fig. 2C). This increase was not statistically significant. The i.m. route was less efficient and resulted in an average of 33 and 14% specific ^{51}Cr release for the HIV and *Plasmodium* epitopes, respectively, at day 10 (Fig. 2B). By 55 days post-infection, the average CTL responses decreased to 23 and 12%, respectively (Fig. 2D). All the above lyses were detected at E:T 100.

To prove formally that the peptide-dependent lysis of MHC class II-deficient P815 cells was carried out by $CD8^+$ lymphocytes, the *in vitro* restimulated splenocytes were panned either on anti-CD4 or anti-CD8 plates prior to the CTL assay. While the cytolytic activities of anti-CD4 panned splenocytes remained unchanged, the anti-CD8 panning resulted in a 2- and 4-fold decrease in the respective HIV- and *Plasmodium* peptide-specific lysis (Fig. 3). The failure to abolish the lytic activity was most probably due to an incomplete removal of the $CD8^+$ T cells from the effector cultures (not shown).

In parallel with the analysis of cytolytic activities, the splenocytes from the MVA.HM-vaccinated animals were assessed in an ELISPOT assay for the frequencies of IFN-γ-producing cells upon peptide stimulation. In this assay, the responses are dependent on the presence of the short $H\text{-}2D^d$- and K^d-restricted peptides and the $CD8^+$ T cells are predominantly responsible for the IFN-γ production (Hanke *et al.*, 1997 *b*). The average frequencies ranged from 40 to 283 IFN-γ-producing cells per 10^6 splenocytes (Table 1). The frequencies correlated well with the detected CTL activities for the more efficient HIV epitope (Fig. 4A), but not for the less efficient *Plasmodium* epitope (Fig. 4B). At 55 days after the i.v. vaccination, there was a decrease in the number of IFN-γ-producing cells, while the specific CTL activities remained similar (Fig. 2C; Table 1). Peptides failed to stimulate detectable IFN-γ production by splenocytes from unprimed animals (data not shown).

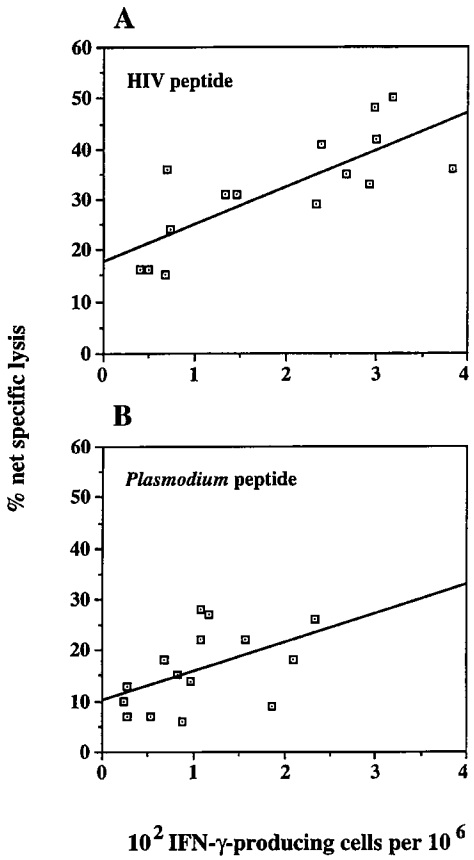

Fig. 4. Correlation of *in vitro* CTL activities with ELISPOT assay frequencies of IFN-γ-producing cells. Experimental data from the MVA.HM i.m. and i.v. 10 day vaccinations were the basis for these graphs. The correlation coefficients *R* involving the HIV (A) and *Plasmodium* (B) peptides were 0·777 and 0·490, respectively.

The MVA-vaccinated animals appeared normal and active up to 55 days post-vaccination, suggesting that neither the injected dose of live attenuated MVA nor the expression of the novel recombinant proteins caused any obvious adverse reactions or discomfort.

Generation of human epitopes in infected CTL targets

A CTL clone and lines for four HLA-restricted HIV epitopes were available and used to test processing and presentation of these epitopes in human cells infected with MVA.H and MVA.HM. All four epitopes were generated, and sensitized infected target cells to CTL lysis (Fig. 5).

Discussion

In the present work, a novel HIV vaccine candidate has been constructed, designed as a multi-CTL epitope protein expressed from live MVA. A single vaccination dose of 10^6 p.f.u. administered i.m. or i.v. elicited CTL responses against both murine epitopes present in the immunogen, the

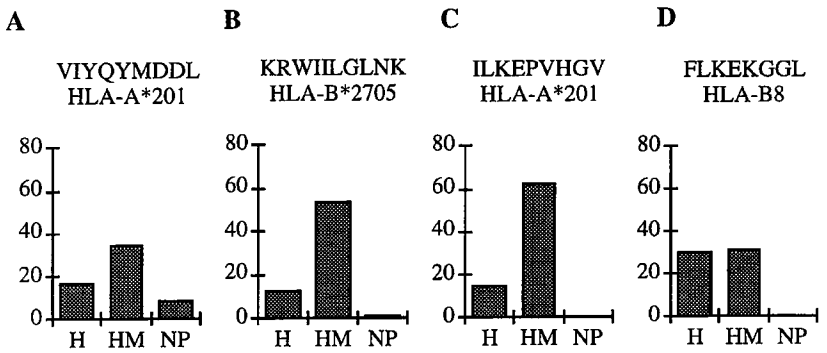

Fig. 5. Generation of human epitopes in MVA.H- and MVA.HM-infected human cells. HLA-matched B lymphoblastoid cell lines were infected with MVA.H or MVA.HM and tested for recognition by an established CTL clone (A) and different cell lines (B–D). The results are shown as percentage specific killing (*y*-axis) and were obtained at effector-to-target ratios of 40:1 (A), 75:1 (B), 2:1 (C) and 5:1 (D). Targets infected with MVA.NP served as a negative control.

i.v. route being more efficient in CTL induction than the i.m. route (Fig. 2). Both MVA.H and MVA.HM induced similar levels of CTL activity against the HIV epitope, and these were comparable to the previously reported CTL induced by MVA expressing influenza virus haemagglutinin and nucleoprotein (Sutter *et al.*, 1994). On the other hand, the *Plasmodium* epitope in MVA.HM-immunized mice elicited consistently lower CTL activities (Figs 2 and 3) and frequencies of peptide-specific IFN-γ-producing cells (Table 1) than the HIV epitope. A number of mechanisms could contribute to this variation in induction of CTL between the two epitopes, which are synthesized *in vivo* in identical cells in equimolar amounts. Firstly, these epitopes are presented by two different H-2^d molecules which may differ in their biochemical and biological properties (Weis & Murre, 1985). Secondly, the efficiency of processing of some, but not other peptides from polyepitope proteins can be affected by their flanking amino acid residues (Hanke *et al.*, 1997*a*; Oldstone *et al.*, 1993; Rabinovich *et al.*, 1994; Ria *et al.*, 1990; Thomson *et al.*, 1995; Whitton *et al.*, 1993). Finally, vaccinia-mediated epitope-specific suppression might have been responsible for the observed differences (T. J. Blanchard and others, unpublished data; Townsend *et al.*, 1988).

A good correlation between CTL activity and the number of cells producing IFN-γ upon peptide stimulation was found for the HIV ($R = 0{\cdot}777$), but not the *Plasmodium* epitope ($R = 0{\cdot}490$; Fig. 4). This might indicate that the malaria epitope was less efficiently presented to CTL *in vivo*. It has recently been shown that extremely low peptide concentrations (10^{-12} to 10^{-15} M) are sufficient to stimulate specific cytotoxic activity, but much higher concentrations ($> 10^{-9}$ M) can be required to drive T cells to IFN-γ production and proliferation (Sad *et al.*, 1996; Valitutti *et al.*, 1996). Alternatively, the MVA/multi-epitope vaccination could have induced high frequency of *Plasmodium*-specific CTL precursors, but the peptide restimulation was inefficient *in vitro* and failed to induce proliferation of a large fraction of the specific memory T cells detected in the ELISPOT assay or lysis in the CTL assay.

A potentially interesting observation is the comparable CTL activity at 10 and 55 days after i.v. administration of MVA.HM, while the number of positive cells in the ELISPOT assay decreased with time (Fig. 3; Table 1). This may indicate that MVA delivered via the i.v. route found cells in which it could more productively replicate and thus increase or prolong the antigen load. Alternatively, antigen-presenting cells could have been reached which presented the antigen more efficiently. The smaller numbers of positive signals in the IFN-γ ELISPOT assay might suggest the presence of mature terminally differentiated CTL responsible for approximately half of the detected cytotoxic activity.

Only a few studies have compared the $CD8^+$ T cell immunogenicity of MVA with other vaccine vectors. MVA was equal to, or more efficient than, the Western Reserve (WR) strain of vaccinia virus in protecting mice against lethal influenza virus (Sutter *et al.*, 1994) or adenocarcinoma tumour line (Carroll *et al.*, 1997) challenges. MVA was also shown to be more efficient than WR or NYVAC strains for boosting of DNA-primed $CD8^+$ T cell responses (J. Schneider and others, unpublished).

Finally, the construction of MVA.HM demonstrated the principle and ease of building complex poly-CTL-epitope vaccines against multiple pathogens. As in HIV infection, there is evidence that specific $CD8^+$ T cell responses may provide a degree of protection against *Plasmodium* malaria (Romero *et al.*, 1989; Hill *et al.*, 1991). Thus, provided the combined HIV/*Plasmodium* polyepitope vaccine induces good CTL responses in humans, it could in theory be useful in certain parts of Africa where the HIV-derived epitopes are relevant for the local HIV-1 clades.

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